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## METHOD OF MODULATING CELL SURVIVAL AND REAGENTS USEFUL FOR SAME

### 5 FIELD OF THE INVENTION

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The present invention relates generally to a method for modulating cell survival. Modulation of cell survival includes inducing, enhancing or otherwise promoting cell survival such as the survival of neural cells as well as facilitating cell death such as the death of targeted cancer cells. The modulation of cell survival is mediated by a region identified on the p75 neurotrophin receptor (p75<sup>NTR</sup>) required for death signalling. The present invention further provides genetic molecules which encode the death signalling region of p75<sup>NTR</sup> which are useful in antagonising death signal function as well as promoting cell death when expressed in targeted cells. The present invention also contemplates recombinant peptides, polypeptides and proteins as well as chemical equivalents, derivatives and homologues thereof which comprise the death signalling portion of p75<sup>NTR</sup>. Particularly useful molecules of the present invention comprise peptides corresponding to soluble forms of the death signalling portion of p75<sup>NTR</sup>. These molecules antagonise p75<sup>NTR</sup>-mediated cell death.

### BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

INSAI

~~The subject specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and~~

a ~~source organism for each nucleotide or amino acid sequence are indicated by~~  
~~information provided in the numeric indicator fields <211>, <212> and <213>,~~  
~~respectively. Nucleotide and amino acid sequences referred to in the specification~~  
~~are defined by the information provided in numeric indicator field <400> followed by~~  
5 ~~the sequence identifier (eg. <400>1, <400>2, etc).~~

The increasing sophistication of recombinant DNA technology is greatly facilitating research and development in the medical and allied health fields. This is particularly the case in the development of recombinant cytokines and growth  
10 factors for use in the treatment of diabetes, acquired immunodeficiency syndrome (AIDS) and a number of cancers.

However, despite this developing knowledge of cytokine and growth factor effector molecules, their full exploitation requires an understanding of the corresponding  
15 cellular receptors and the complex biochemical and physiological signalling pathways initiated following interaction with ligands or following other stimulation such as disease, receptor aggregation or trauma.

A number of soluble trophic factors have been shown to exhibit an effect on neural  
20 survival *in vivo*. Many of these factors act directly on the developing neuron within, for example, the dorsal root ganglia (DRG). One factor of particular importance is nerve growth factor (NGF) [1]. The p75 neurotrophin receptor (hereinafter referred to as "p75<sup>NTR</sup>"), which is capable of associating with trk growth factor receptors, facilitates high affinity NGF binding and survival signalling. Although NGF has been  
25 proposed as a potential therapeutic molecule to promote survival of neurons, NGF is a multifunctional molecule and its pleiotrophy may adversely effect a range of non-neural cells.

p75<sup>NTR</sup> is also multifunctional. It has now been shown that p75<sup>NTR</sup> is capable of  
30 acting as a death receptor. Elevated p75<sup>NTR</sup> expression results in increased cell death *in vitro* and *in vivo* [2-4]. Furthermore, down-regulation of p75<sup>NTR</sup> prevents

neural death after growth-factor withdrawal or axotomy [5, 6]. Consistent with the dual functions of p75<sup>NTR</sup>, mice with deleted p75<sup>NTR</sup> genes have a dramatic reduction of NGF dependent neurons, such as dorsal root ganglia, but increased numbers of other neuron populations (sympathetic and basal forebrain neurons) suggesting  
5 lack of naturally occurring cell death [7, 8]. p75<sup>NTR</sup> is also implicated in mediating death of neural, oligodendrocytes and Schwann cells [8, 9].

p75<sup>NTR</sup> is a member of the tumor necrosis factor (TNF) receptor/Fas superfamily, showing homology not only to the extracellular ligand binding domain but also to a  
10 cytoplasmic motif known as the "death domain", so termed because of the cytotoxic actions of proteins containing the domain [9].

There is an accumulating body of evidence which suggests that p75<sup>NTR</sup> is involved in mediating cell death in a variety of degenerative diseases. During adulthood,  
15 p75<sup>NTR</sup> expression is down-regulated in most brain areas but is rapidly induced in ischemia (stroke) and results in transient increased p75<sup>NTR</sup> expression and apoptosis, as do both peripheral and motor nerve lesions [10-12]. p75<sup>NTR</sup> is also up regulated in patients with MND [13], and in experimental allergic encephalomyelitis (a model of multiple sclerosis; [14]). Intriguingly, in the basal forebrain and  
20 hippocampus, areas involved in learning and memory, p75<sup>NTR</sup> is highly expressed in aged rodents and in Alzheimer's patients, where extensive neural death is occurring [15, 16]. These data suggest that p75<sup>NTR</sup> is involved not only in normal developmental cell death, but may mediate the cell death occurring after injury or in neurodegenerative disease.

25 In work leading up to the present invention, the inventors sought to elucidate the region on p75<sup>NTR</sup> which mediates death signalling. The inventors surprisingly determined that the death signal is not the cytoplasmic motif known as the death domain [9] but is a region adjacent the membrane domain on p75<sup>NTR</sup>. The  
30 identification of this region provides for an opportunity to modulate cell survival by antagonising the death signalling region or promoting apoptosis by providing cells

with the genetic material to express the death signalling region adjacent, proximal or otherwise juxtaposed or associated with the membrane or to express the death signalling region in multimeric form.

## 5 SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers  
10 but not the exclusion of any other element or integer or group of elements or integers.

One aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides  
15 which encodes an amino acid sequence which is capable of signalling, inducing or otherwise facilitating the death of a cell in which said amino acid sequence is adjacent, proximal or otherwise juxtaposed to the membrane of said cell or when said amino acid sequence is in multimeric form.

20 Another aspect of the present invention is directed to a nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides which encodes a peptide, polypeptide or protein capable of signalling, inducing or otherwise facilitating death of a cell in which it is expressed wherein said peptide, polypeptide or protein comprises a membrane associating portion and/or a  
25 multimer-forming portion and a portion which corresponds to all or part of the cytoplasmic region of p75<sup>NTR</sup> or a functional equivalent, derivative or homologue thereof.

Yet another aspect of the present invention contemplates homologues, analogues  
30 and derivatives of a nucleic acid molecule which encodes a peptide, polypeptide or protein which is capable of signalling inducing or otherwise facilitating death of a

cell in which it is expressed wherein said peptide, polypeptide or protein comprises a membrane associating portion and/or a multimer-forming portion and a portion which corresponds to all or part of the cytoplasmic region of p75<sup>NTR</sup> or a functional equivalent, derivative or homologue thereof.

5

A further aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which encodes an amino acid sequence which inhibits or reduces p75<sup>NTR</sup>-mediated cell death wherein said amino acid sequence is a soluble form of the p75<sup>NTR</sup> receptor corresponding to an intracellular  
10 region adjacent, proximal or otherwise juxtaposed to the membrane of said cell.

~~Still another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence which is substantially as set forth in <400>3 or is a nucleotide sequence capable of  
15 hybridising to <400>3 or its complementary form under low stringency conditions or is a nucleotide sequence having at least 60% identity to <400>3.~~

~~Still yet another aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence or a complementary form thereof,  
20 which nucleotide sequence encodes an amino acid sequence substantially as set forth in <400>4 or a derivative, homologue or chemical equivalent thereof or an amino acid sequence having at least 60% identity thereto.~~

Even yet another aspect of the present invention provides a genetic construct  
25 comprising an isolated nucleic acid molecule which comprises a sequence of nucleotides which corresponds or is complementary to a death signal region from p75<sup>NTR</sup> or a homologue, analogue or derivative thereof.

Another aspect of the present invention contemplates an isolated peptide,  
30 polypeptide or protein comprising the cytoplasmic region of p75<sup>NTR</sup> which signals, induces or otherwise facilitates cell death when said peptide, polypeptide or protein

is adjacent, proximal or otherwise juxtaposed to a membrane-associating region such as from p75<sup>NTR</sup> or other membrane molecule and/or said peptide, polypeptide or protein is capable of forming multimers or a derivative, homologue, chemical equivalent or analogue of said peptide, polypeptide or protein. This aspect of the  
5 present invention does not extend to the full length p75<sup>NTR</sup>.

Still another aspect of the present invention contemplates a method for inhibiting, reducing or otherwise antagonising a p75<sup>NTR</sup>-mediated death signal in a neural cell, said method comprising introducing a nucleic acid molecule capable of being  
10 expressed to an expression product which corresponds to a non-membrane associated form of the p75<sup>NTR</sup> death signal region or a derivative, functional equivalent or homologue thereof.

Yet another aspect of the invention contemplates a method for inhibiting, reducing  
15 or otherwise antagonising a p75<sup>NTR</sup>-mediated death signal in a neural cell, said method comprising contacting a cell carrying a p75<sup>NTR</sup> with a death signal-inhibiting effective amount of a molecule capable of antagonising the death signal of p75<sup>NTR</sup> or a component of the death signalling pathway.

20 Even still another aspect of the present invention provides a biological composition comprising a genetic molecule capable of expressing a p75<sup>NTR</sup> death signal antagonist or a p75<sup>NTR</sup> death signal.

Another aspect of the present invention is directed to a biological composition  
25 comprising a molecule capable of antagonising p75<sup>NTR</sup>-mediated death signalling of a cell.

Yet still another aspect of the present invention contemplates a method for modulating p75<sup>NTR</sup>-mediated death signal in a neural cell, said method comprising  
30 administering an agent which antagonises or agonises cleavage of the extracellular domain of p75<sup>NTR</sup>.

Still another aspect of the present invention provides a method for inhibiting, reducing or otherwise antagonising p75<sup>NTR</sup>-mediated death signal in a neural cell, said method comprising administering a peptide, polypeptide or protein or analogues or mimetics thereof which correspond to a non-membrane associated  
5 form of the p75<sup>NTR</sup> death signal region or a derivative, functional equivalent or homologue thereof.

Another aspect of the present invention provides peptide antagonists of the p75<sup>NTR</sup> death signal or functional analogues or mimetics thereof.

10

~~FIG 3~~ ~~183~~ The terms "c35" and "35mer" are used interchangeably herein to refer to 35-amino acid domain juxtaposed to the membrane. When in soluble form, this peptide is referred to as soluble c35 or 35mer. The nucleotide and amino acid sequence of c35 are shown in <400>7 and <400>8, respectively. The term "29mer" refers to a  
15 truncated form of the 35mer. Six amino acids have been deleted from the C-terminal end. The nucleotide and amino acid sequence of 29mer are shown in <400>11 and <400>12, respectively. The present invention extends to isolated forms of c35 and the 29mer, to compositions comprising same and to genetic sequences encoding same.

20

## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1** is a diagrammatic representation showing plasmid constructs with and without the death signalling region. The black region is the putative "death domain" [9] but which is not directly involved in p75<sup>NTR</sup> mediated cell death.

**Figure 2** is a graphical representation showing survival of DRG neurons 17 hours after microinfection and cultured in LIF. The data show that the amino acid domain juxtaposed to the membrane is required for death signalling rather than the putative "death domain" [9].

**Figure 3** is a graphical representation showing DRG survival 16 hours after microinjection and cultured in LIF. The data show that over 90% of cells die when expressing the death signal linked to the membrane.

**Figure 4** is a graphical representation showing DRG survival 20 hours after microinjection and cultured in LIF. These data show that when the death signal is not associated with the membrane, that the ability to induce death is removed.

**Figure 5** is a graphical representation showing that the c35 soluble protein (i.e. p75<sup>NTR</sup> death signal region) inhibits death signalling mediated by p75<sup>NTR</sup>.

**Figure 6** is a graphical representation showing that soluble c35 inhibits p75<sup>NTR</sup>-mediated death signalling.





**Figure 7** is a graphical representation showing protection of membrane-bound killing-domain by a soluble 35 amino acid peptide and a soluble 29 amino acid peptide. The cells were subjected to microinjection of sptc35 or GFP followed 30 minutes later by either peptide c35 or the 29mer peptide.

**Figure 8** is a graphical representation showing that peptide 29 which has a



palmitoyl group at the membrane (amino) end and which facilitates association with the membrane mediates to cell death. In contrast, the soluble 35 amino acid molecule tends to protect the cells. F, Fluoro tagged; pen, penetratin.

5 **Figure 9** is a graphical representation showing the palmitoylated 29mer fused to penetratin mediates specific killing whereas non-palmitoylated 29mer blocks cell death. Cells were treated with 2  $\mu$ M peptide for 1-2 hours then washed. pen, penetratin, F29, 29 mer; Palm, palmitoylation.

- 10
-  pen F29
  -  pen F29 Palm
  -  pen gp130 Palm
  -  pen

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

1/5B4  
1B4  
The present invention arose in part following an investigation of the neurotrophin receptor, p75<sup>NTR</sup>, in its capacity as a death signalling protein. Although the p75<sup>NTR</sup> molecule comprises a putative death domain [9], in accordance with the present invention, this death domain is not directly associated with p75<sup>NTR</sup>-mediated cell death. Rather, a region adjacent, proximal or otherwise juxtaposed to the membrane domain of p75<sup>NTR</sup> is required for cell death. The nucleotide and corresponding amino acid sequence of the death domain [9] is shown in <400>9 and <400>10, respectively.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides which encode an amino acid sequence which is capable of signalling, inducing or otherwise facilitating the death of a cell in which said amino acid sequence is adjacent, proximal or otherwise juxtaposed to the membrane of said cell or when said amino acid sequence is in multimeric form.

Reference herein to the signalling, inducing or otherwise facilitating the death of a cell or a death signal is meant to be construed in its broadest sense meaning that the amino acid sequence plays a role in a pathway leading to cell death. The death signal may also be regarded as an apoptotic signal. Although not wishing to limit the present invention to any one theory or mode of action, it is proposed herein that there is a pathway from p75<sup>NTR</sup> activation to caspase activation and cellular degeneration. p75<sup>NTR</sup>-mediated cell death may also occur directly or indirectly via Bcl-2.

The present specification refers interchangeably to death signal, death signal region, signalling, inducing or otherwise facilitating the death of a cell and c35.

The nucleic acid molecule of the present invention may encode a non-full length

p75<sup>NTR</sup> molecule although to facilitate cell death, the nucleic acid molecule must encode all or part of the cytoplasmic portion of the p75<sup>NTR</sup> molecule and a sufficient amount of the membrane domain such that the region referred to herein as the death signal is membrane associated. A "part" of the cytoplasmic domain of p75<sup>NTR</sup> includes all or a death-inducing functional part of a 35 amino acid region juxtaposed to the membrane domain. An example of a part of the 35 amino acid region is a truncated form. One such form is referred to herein as the "29 mer". Alternatively, the cytoplasmic domain of the p75<sup>NTR</sup> molecule is in multimeric form or capable of forming multimers. A multimer comprises two or more copies of the molecule such as a dimer, trimer or larger copy molecule.

The term "membrane associated" means that the death signal is adjacent, proximal or otherwise juxtaposed to the membrane of a cell expressing the nucleic acid molecule.

The "death signal region" and other related terms are used herein to describe functionally the region of the cytoplasmic portion of p75<sup>NTR</sup> which is adjacent, proximal or otherwise juxtaposed to a region of p75<sup>NTR</sup> which associates with the membrane or which cytoplasmic portion is in multimeric form. The death signal region is not the same portion of the molecules as the "death domain" [9] although there may be functional similarities in death signalling.

Accordingly, another aspect of the present invention is directed to a nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides which encodes a peptide, polypeptide or protein capable of signalling, inducing or otherwise facilitating death of a cell in which it is expressed wherein said peptide, polypeptide or protein comprises a membrane associating portion and/or a multimer-forming portion and a portion which corresponds to all or part of the cytoplasmic region of p75<sup>NTR</sup> or a functional equivalent, derivative or homologue thereof.

In order to signal, induce or otherwise facilitate death of a cell, the death signal region is preferably adjacent, proximal or otherwise juxtaposed to the cell membrane. This may be facilitated by modifying a peptide such that it associates with the membrane. One example of this type of modification is palmitoylation.

5 This puts a palmitoyl group at the membrane (amino) end of the peptide.

Accordingly, another aspect of the present invention contemplates palmitoylated peptides, polypeptides or proteins comprising all or part of the death signal region of p75<sup>NTR</sup>. Such peptides are particularly useful in promoting cell death.

10

The present invention also extends to multimeric forms of death signal peptides, polypeptides and proteins and attachments which facilitate same. A multimer comprises two or more molecules. The present invention also extends to cleavage forms of the full length p75<sup>NTR</sup> molecule.

15

In one embodiment, the membrane portion is derived from p75<sup>NTR</sup> or a functional equivalent, derivative or homologue thereof. In another embodiment, the membrane domain is from another molecule such as a receptor or other ligand-binding molecule. Examples of receptors according to this aspect of the present invention include cytokine receptors (e.g. the Leukaemia Inhibitory Factor (LIF) receptor, interleukin receptor, and colony-stimulating factor receptors). Examples of ligand-binding molecules include immunoglobulins and T cell receptors.

20 When in multimeric form, the molecule is only optionally associated with the membrane to effect cell death.

The nucleic acid molecule may comprise cDNA or genomic DNA or may comprise ribonucleotides such as mRNA. The nucleic acid molecule may be derived from a cDNA or genomic molecule encoding p75<sup>NTR</sup> or a derivative or homologue thereof or may be prepared by the stepwise addition of nucleotides in a defined sequence.

30

The nucleic acid molecule of the present invention may also be considered as corresponding to a "gene".

Reference herein to a "gene" is to be taken in its broadest context and includes:

- 5 (i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences);
- (ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) optionally comprising 5'- or 3'-untranslated sequences of the gene; or
- 10 (iii) an amplified DNA fragment or other recombinant nucleic acid molecule produced *in vitro* and comprising all or a part of the coding region and/or 5'- or 3'-untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all  
15 or part of a functional product. A functional product is one which comprises a sequence of nucleotides or is complementary to a sequence of nucleotides which encodes a functional death signal from p75<sup>NTR</sup> or its derivative or homologue.

The nucleotide sequence of the present invention may correspond to the cDNA or  
20 genomic sequence of a gene encoding p75<sup>NTR</sup> or a death signal region thereof or may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions. Nucleotide insertional derivatives of the nucleic acid molecule of the present invention include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional  
25 nucleotide sequence variants are those in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one  
30 nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not

change the amino acid defined by the codon. Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity.

- 5 Accordingly, another aspect of the present invention contemplates homologues, analogues and derivatives of a nucleic acid molecule which encodes a peptide, polypeptide or protein which is capable of signalling, inducing or otherwise facilitating death of a cell in which it is expressed wherein said peptide, polypeptide or protein comprises a membrane associating portion and/or multimer-forming
- 10 portion and a portion which corresponds to all or part of the cytoplasmic region of p75<sup>NTR</sup> or a functional equivalent, derivative or homologue thereof.

For the present purpose, "homologues" of a nucleic acid molecule as herein defined or of a nucleotide sequence shall be taken to refer to an isolated nucleic

15 acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

- 20 "Analogues" of a nucleic acid molecule as herein defined or of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated
- 25 nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a nucleic acid molecule as herein defined or of a nucleotide

30 sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part

- thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5' and 3' terminal fusions as well as
- 5 intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional
- 10 variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.
- 15 In one embodiment, the derivatives encode a peptide, polypeptide or protein which induces cell death. In another embodiment, the derivatives do not induce cell death but antagonise the death signal.

According to this latter embodiment, there is provided an isolated nucleic acid

20 molecule comprising a sequence of nucleotides which encodes an amino acid sequence which inhibits or reduces p75<sup>NTR</sup>-mediated cell death wherein said amino acid sequence is a soluble form of the p75<sup>NTR</sup> receptor corresponding to an intracellular region adjacent, proximal or otherwise juxtaposed to the membrane of said cell.

25

The nucleic acid molecule of the present invention may be based on a nucleotide sequence of the gene or cDNA encoding p75<sup>NTR</sup> from any animal such as from mammals. Preferred mammals include humans, primates, livestock animals (e.g. cows, sheep, horses, pigs, donkeys, goats), laboratory test animals (e.g. rabbits,

30 mice, rats, guinea pigs, hamsters), companion animals (e.g. dogs, cats) and captive wild animals.

A particularly preferred sequence is from human or primate or murine p75<sup>NTR</sup>.

Although not wishing to limit the present invention to any one theory or mode of action, it is proposed that the extracellular domain of p75<sup>NTR</sup> may be cleaved off  
5 resulting in active death signal (see Zupan *et al* [20]). Accordingly, by antagonising cleavage, cell death may be prevented or at least delayed or inhibited. Conversely, for targeted cancer cells, an agonist of p75<sup>NTR</sup> extracellular domain cleavage would promote cell death.

10 Accordingly, another aspect of the present invention contemplates a method for modulating p75<sup>NTR</sup>-mediated death signal in a neural cell, said method comprising administering an agent which antagonises or agonises cleavage of the extracellular domain of p75<sup>NTR</sup>.

15 Preferably, to prevent neural cell death, extracellular p75<sup>NTR</sup> cleavage is antagonised.

The present invention is exemplified using a nucleotide sequence from rat p75<sup>NTR</sup> cDNA. This is done, however, with the understanding that the nucleotide sequence  
20 may be from p75<sup>NTR</sup> genomic or cDNA from any animal.

*IWS B5* 1. B5 ~~Accordingly, another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence or a complementary form thereof wherein said nucleotide sequence is capable of hybridising to <400>1 or a  
25 complementary form thereof under low stringency conditions, such as at 42 °C.~~

~~The nucleotide sequence set forth in <400>1 is the cDNA sequence encoding p75<sup>NTR</sup>. The nucleic acid molecule according to this aspect of the present invention does not extend to the full length p75<sup>NTR</sup> cDNA sequence but comprises a portion  
30 which encodes an amino acid sequence which signals, induces or otherwise facilitates cell death when associated with a membrane portion of p75<sup>NTR</sup> or other~~



~~molecules.~~

~~Accordingly, another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence which is substantially as set forth in <400>7 or is a nucleotide sequence capable of hybridising to <400>7 or a complementary form thereof under low stringency conditions such as at 42 °C or is a nucleotide sequence having at least 60% identity to <400>7.~~

~~The nucleotide sequence set forth in <400>7 is the death signal defined herein associated with p75<sup>NTR</sup>. This sequence encodes a 35 amino acid region also referred to herein as "c35". Truncated forms of c35 are also contemplated by the present invention such as a 25-30 amino acid molecules. One particular example is a 29mer which lacks carboxy terminal amino acids 30 to 35. As stated above, the present invention extends to palmitoylated c35 and its derivatives as well as molecules fused with molecules to facilitate membrane passage such as penetratin and the TAT protein from HIV.~~

Reference herein to a low stringency such as at 42°C includes and encompasses from at least about 0% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions. Preferably, low stringency is determined at 42°C.

INSB6  
1.96 ~~The present invention further contemplates a nucleic acid molecule comprising a nucleotide sequence or a complementary form thereof, which nucleotide sequence encodes an amino acid sequence substantially as set forth in <400>8 or a derivative, homologue or chemical equivalent thereof or an amino acid sequence having at least 60% identity thereto.~~

~~The amino acid sequence of <400>8 corresponds to the amino acid sequence of the p75<sup>NTR</sup> death signal.~~

- 10 The term "identity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, the term "similarity" may also be used and includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels.
- 15 levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino
- 20 acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the alignment method of Needleman and Wunsch [17]. Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP
- 25 is available on ANGIS (Australian National Genomic Information Service) at website <http://mel1.angis.org.au..>

The present invention further comprises a nucleic acid molecule comprising the nucleotide sequence:

- 30  $\{n_1 \dots n_x\}_a \{n'_1 \dots n'_y\}_c \text{ a } \{n''_1 \dots n''_z\}_d$   
wherein

$\{n_1 \dots n_x\}$  is a sequence of  $x$  nucleotides encoding an extracellular portion of a receptor or ligand-binding molecule;

5  $\{n'_1 \dots n'_y\}$  is a sequence of  $y$  nucleotides encoding a transmembrane peptide, polypeptide or protein or a molecule capable of inducing multimerisation;

10  ~~$\{n''_1 \dots n''_z\}$  is a sequence of  $z$  nucleotides comprising a nucleotide sequence substantially as set forth in <400>7 or a nucleotide sequence encoding an amino acid sequence substantially as set forth in <400>8 or a nucleotide sequence capable of hybridising to <400>7 or a complementary form thereof under low stringency conditions such as at 42 °C or a nucleotide sequence having at least 60% identity to <400>7;~~

15 b, c and d may be the same or different and each is 0, 1 or >1;

x, y and z may be the same or different and each is 0, 1 or >1;

a is a nucleotide bond;

20

wherein when c is 1 or >1 and d is 1 or >1 and wherein when the molecule is expressed in a neural cell, the expression product signals, induces or otherwise facilitates cell death.

25 ~~Preferably,  $\{n_1 \dots n_x\}$  comprises the nucleotide sequence substantially as set forth in <400>3 or is a nucleotide sequence having at least about 60% identity thereto or is capable of hybridising to <400>3 or its complementary form under low stringency conditions such as at 42 °C.~~

30 Preferably,  $\{n'_1 \dots n'_y\}$  comprises the nucleotide sequence substantially as set forth in <400>5 or is a nucleotide sequence having at least about 60% identity thereto or

is capable of hybridising to <400>5 or its complementary form under low stringency conditions such as at 42 °C.

The nucleotide sequences  $\{n_1 - - - n_x\}$ ,  $\{n'_1 - - - n'_y\}$  and  $\{n''_1 - - - n''_z\}$  may be in any  
5 order and in any combination.

For the production of a recombinant peptide, polypeptide or protein comprising the death signal, the nucleic acid molecule of the present invention is placed, in the sense orientation, in operable connection with a suitable promoter sequence and  
10 introduced into a suitable expression system, for example a bacterial, yeast, baculovirus, plant, animal or other expression system.

Accordingly, a further aspect of the present invention provides a genetic construct comprising an isolated nucleic acid molecule which comprises a sequence of  
15 nucleotides which corresponds or is complementary to a death signal region from p75<sup>NTR</sup> or a homologue, analogue or derivative thereof.

According to this embodiment, the coding region of the death signal from p75<sup>NTR</sup> may be placed in operable connection with a promoter sequence such that a gene  
20 product is capable of being expressed under the control of said promoter sequence.

Optionally, said genetic construct further comprises a terminator sequence.

In the present context, the term "in operable connection with" is used to indicate  
25 that expression of the isolated nucleotide sequence is under the control of the promoter sequence with which it is connected.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA  
30 sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active

in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the genetic constructs of the present invention include the SV40 polyadenylation signal, amongst others.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation in eukaryotic cells, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers). For expression in prokaryotic cells, such as bacteria, the promoter should at least contain the -35 box and -10 box sequences.

- 15 A promoter is usually, but not necessarily, positioned upstream or 5', of the nucleotide sequence encoding the death signal of p75<sup>NTR</sup>, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.
- 20 In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of an isolated nucleic acid molecule, in a cell, such as a plant, animal, insect, fungal, yeast or bacterial cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of a nucleic acid
- 25 molecule which expression it regulates and/or to alter the spatial expression and/or temporal expression of same. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule, thereby conferring copper inducibility on the expression of said molecule.

- Placing an isolated nucleic acid molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural
- 5 gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the
- 10 preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.
- 15 Examples of promoters suitable for use in genetic constructs of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant, animal, insect, fungal, yeast or bacterial cells. The promoter may regulate the expression of the nucleic acid molecule constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to
- 20 the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or plant pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a nucleic acid

25 molecule in a yeast or bacterial cell.

Examples of preferred promoters include the bacteriophage T7 promoter, bacteriophage T3 promoter, SP6 promoter, *lac* promoter, *tac* promoter, SV40 early promoter, and the like.

30

The genetic construct contemplated herein is introduced into a suitable expression

system for a time and under conditions sufficient for expression of said death signal or inhibitor portion from p75<sup>NTR</sup> to occur.

The genetic construct may also comprise a nucleotide sequence corresponding to  
5 all or part of the membrane domain of p75<sup>NTR</sup> or other membrane molecules.

Accordingly, a further aspect of the invention contemplates a recombinant peptide, polypeptide or protein produced by expressing the isolated nucleic acid molecule herein described in a suitable host cell. The present invention extends also to a  
10 synthetic peptide fragment of said recombinant gene product.

The present invention further contemplates an isolated peptide, polypeptide or protein comprising the cytoplasmic region of p75<sup>NTR</sup> which signals, induces or otherwise facilitates cell death when said peptide, polypeptide or protein is  
15 adjacent, proximal or otherwise juxtaposed to a membrane-associating region such as from p75<sup>NTR</sup> or other membrane molecule and/or is in multimeric form or a derivative, homologue, chemical equivalent or analogue of said peptide, polypeptide or protein. This aspect of the present invention does not extend to the full length p75<sup>NTR</sup>.

20 Suitable molecules according to this aspect of the present invention include a peptide, polypeptide or protein corresponding to a soluble form of the death signalling region of p75<sup>NTR</sup> or a molecule capable of antagonising that region or a component of the death signalling pathway. An example of a possible component  
25 of the death signalling pathway is Bcl-2.

The peptide, polypeptide or protein of this aspect of the present invention is useful *inter alia* as a therapeutic molecule to antagonise p75<sup>NTR</sup>-mediated death signalling. For example, the peptide, polypeptide or protein may themselves be  
30 administered to directly antagonise p75<sup>NTR</sup>-mediated death signalling or the peptide, polypeptide or protein may need to be chemically modified to facilitate penetration

into the cell. One such chemical modification is fusion to or co-expression with penetratin or the TAT protein from HIV. Alternatively, the death signalling region of p75<sup>NTR</sup> may be used to screen for antagonists of this region. Such antagonists may, for example, be identified following natural product screening or the screening of chemical libraries. For natural product screening suitable environments include, but are not limited to, plants, bacteria and other microorganisms, river and sea beds, coral and arctic or antarctic regions. The present invention also contemplates antagonists directed to other components of the p75<sup>NTR</sup>-mediated death signalling pathway. Such components to be targeted include but are not limited to Bcl-2 or related or homologous molecules. Preferably, for peptides, polypeptides and proteins designed to induce cell death, the molecules are palmitoylated.

~~1.39~~ 1.39 ~~Preferably, the peptide, polypeptide or protein comprises an amino acid sequence substantially as set forth in <400>8 or an amino acid sequence having at least 60% identity thereto or a chemical equivalent, derivative, homologue or analogue of said peptide, polypeptide or protein.~~

The term "isolated" means that the peptide, polypeptide or protein of the present invention is provided in a form which is distinct from that which occurs in nature, preferably wherein one or more contaminants have been removed. Accordingly, the isolated peptide, polypeptide or protein of the invention may be partially-purified or substantially pure, in which a substantial amount of the contaminants have been removed or in sequencably pure or substantially homogeneous form.

25

The term "sequencably pure" means that the isolated peptide, polypeptide or protein is provided in a form which is sufficiently purified to facilitate amino acid sequence determination using procedures known to those skilled in the art.

30 The term "substantially homogeneous" means that the isolated peptide, polypeptide or protein of the present invention is at least about 95% free of



contaminants, more preferably at least about 99% free of contaminants, including 100% purity.

The present invention extends to a range of derivatives and chemical analogues of the peptide, polypeptide or protein.

Furthermore, the amino acids of a homologous polypeptide may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

10

~~"Analogues" encompass death signal containing peptides, polypeptides or proteins which are at least about 60% identical to the p75<sup>NTR</sup> death signal sequence <400>8], notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein. "Analogues" also encompass polypeptide mimotypes.~~

15

The term "derivative" in relation to a peptide, polypeptide or protein shall be taken to refer hereinafter to mutants, parts or fragments derived from the functional p75<sup>NTR</sup> molecule or death signal region thereof or derivatives thereof which may or may not possess the death signal activity of the functional p75<sup>NTR</sup>. Derivatives include modified peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, derivatives of the peptide, polypeptide or protein described herein comprise fragments or parts of an amino acid sequence disclosed herein are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject polypeptides. Procedures for derivatizing peptides are well-known in the art.

30

~~A homologue, analogue or derivative of <400>2 or <400>8 may comprise an amino~~

~~acid substitution or said <400> 2 or 8 may encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as~~  
a "conservative", in which case an amino acid residue contained in a phospholipase  
5 inhibitory protein is replaced with another naturally-occurring amino acid of similar character, for example Gly ↔ Ala, Val ↔ Ile ↔ Leu, Asp ↔ Glu, Lys ↔ Arg, Asn ↔ Gln or Phe ↔ Trp ↔ Tyr.

Substitutions encompassed by the present invention may also be "non-  
10 conservative", in which an amino acid residue which is present in a phospholipase inhibitory protein is substituted with an amino acid having different properties, such as a naturally-occurring amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid.

15 Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

Naturally-occurring amino acids include those listed in Table 1. Non-conventional  
20 amino acids encompassed by the invention include, but are not limited to those listed in Table 2.

Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the  
25 N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

TABLE 1

5	Amino Acid	Three-letter Abbreviation	One-letter Symbol
	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
10	Aspartic acid	Asp	D
	Cysteine	Cys	C
	Glutamine	Gln	Q
	Glutamic acid	Glu	E
	Glycine	Gly	G
15	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
20	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
25	Tyrosine	Tyr	Y
	Valine	Val	V
	Any amino acid as above	Xaa	X

TABLE 2

	Non-conventional amino acid	Code	Non-conventional amino acid	Code
5	$\alpha$ -aminobutyric acid	Abu	L-N-methylalanine	Nmala
	$\alpha$ -amino- $\alpha$ -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
	aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
			L-N-methylaspartic acid	Nmasp
10	aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
	aminonorbomyl- carboxylate	Norb	L-N-methylglutamine	Nmgln
			L-N-methylglutamic acid	Nmglu
	cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
	cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15	D-alanine	Dal	L-N-methylleucine	Nmleu
	D-arginine	Darg	L-N-methyllysine	Nmlys
	D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
	D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
	D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20	D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
	D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
	D-isoleucine	Dile	L-N-methylproline	Nmpro
	D-leucine	Dleu	L-N-methylserine	Nmser
	D-lysine	Dlys	L-N-methylthreonine	Nmthr
25	D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
	D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
	D-phenylalanine	Dphe	L-N-methylvaline	Nmval
	D-proline	Dpro	L-N-methylethylglycine	Nmetg
	D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
30	D-threonine	Dthr	L-norleucine	Nle
	D-tryptophan	Dtrp	L-norvaline	Nva

	D-tyrosine	Dtyr	$\alpha$ -methyl-aminoisobutyrate	Maib
	D-valine	Dval	$\alpha$ -methyl- $\gamma$ -aminobutyrate	Mgab
	D- $\alpha$ -methylalanine	Dmala	$\alpha$ -methylcyclohexylalanine	Mchexa
	D- $\alpha$ -methylarginine	Dmarg	$\alpha$ -methylcyclopentylalanine	Mcpen
5	D- $\alpha$ -methylasparagine	Dmasn	$\alpha$ -methyl- $\alpha$ -naphthylalanine	Manap
	D- $\alpha$ -methylaspartate	Dmasp	$\alpha$ -methylpenicillamine	Mpen
	D- $\alpha$ -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- $\alpha$ -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D- $\alpha$ -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Nom
10	D- $\alpha$ -methylisoleucine	Dmile	N-amino- $\alpha$ -methylbutyrate	Nmaabu
	D- $\alpha$ -methyllleucine	Dmleu	$\alpha$ -naphthylalanine	Anap
	D- $\alpha$ -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- $\alpha$ -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D- $\alpha$ -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
15	D- $\alpha$ -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- $\alpha$ -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- $\alpha$ -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- $\alpha$ -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D- $\alpha$ -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
20	D- $\alpha$ -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
	D- $\alpha$ -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
25	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl) glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl) glycine	Nbhe

	D-N-methylglutamine	Dnmgin	N-(3-guanidinopropyl) glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
5	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl) glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
10	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
	D-N-methylomithine	Dnmom	N-methylcyclopentylalanine	Nmcpn
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
15	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl-α-naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ-aminobutyric acid	Gabu	N-(p-hydroxyphenyl)glycine	Nhtyr
20	L-t-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
	L-α-methylaspartate	Masp	L-α-methyl-t-butylglycine	Mtbug
25	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
	L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
	L-α-methylhistidine	Mhis	L-α-methylhomo phenylalanine	Mhphe
	L-α-methylisoleucine	Mile	N-(2-methylthioethyl) glycine	Nmet
30	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys

L- $\alpha$ -methionine	Mmet	L- $\alpha$ -methylnorleucine	Mnle
L- $\alpha$ -methylnorvaline	Mnva	L- $\alpha$ -methylornithine	Morn
L- $\alpha$ -methylphenylalanine	Mphe	L- $\alpha$ -methylproline	Mpro
L- $\alpha$ -methyserine	Mser	L- $\alpha$ -methylthreonine	Mthr
5 L- $\alpha$ -methyltryptophan	Mtrp	L- $\alpha$ -methyltyrosine	Mtyr
L- $\alpha$ -methylvaline	Mval	L-N-methylhomo	
		phenylalanine	Nmhph
N-(N-(2,2-diphenylethyl)		N-(N-(3,3-diphenylpropyl)	
carbonylmethyl)glycine	Nnbhm	carbonylmethyl)glycine	Nnbhe
10 1-carboxy-1-(2,2-diphenyl-			
ethylamino)cyclopropane	Nmbc		

The present invention provides therefore, peptides, polypeptides and proteins  
 15 which inhibit p75<sup>NTR</sup> death signalling and/or cleavage of extracellular domain of  
 p75<sup>NTR</sup>.

Accordingly, another aspect of the present invention contemplates a method for  
 inhibiting, reducing or otherwise antagonising p75<sup>NTR</sup>-mediated death signal in a  
 20 neural cell, said method comprising administering a peptide, polypeptide or protein  
 or analogues or mimetics thereof which correspond to a non-membrane associated  
 form of the p75<sup>NTR</sup> death signal region or a derivative, functional equivalent or  
 homologue thereof.

25 Yet another aspect of the present invention is directed to peptide antagonists of the  
 p75<sup>NTR</sup> death signal or functional analogues or mimetics thereof.

The present invention provides for a method of treatment or prophylaxis of disease  
 conditions associated with neural death or where cell death is to be promoted such  
 30 as in treating or preventing cancer growth and/or development.

In one embodiment, it has been determined in accordance with the present invention that expression of a nucleic acid molecule encoding only death signal and not adjacent, proximal or juxtaposed to a membrane-associating sequence results in antagonising of the death signal.

5

According to this embodiment, the present invention contemplates a method for inhibiting, reducing or otherwise antagonising a p75<sup>NTR</sup>-mediated death signal in a neural cell, said method comprising introducing a nucleic acid molecule capable of being expressed to an expression product which corresponds to a non-membrane  
10 associated form of the p75<sup>NTR</sup> death signal region or a derivative, functional equivalent or homologue thereof.

In a related embodiment there is provided a method for inhibiting, reducing or otherwise antagonising a p75<sup>NTR</sup>-mediated death signal in a neural cell, said  
15 method comprising contacting a cell carrying a p75<sup>NTR</sup> with a death signal-inhibiting effective amount of a molecule capable of antagonising the death signal of p75<sup>NTR</sup> or a component of the death signalling pathway.

This aspect of the present invention is useful for the treatment of a range of  
20 neurodegenerative diseases such as cerebral palsy, trauma induced paralysis, vascular ischaemia associated with stroke, neural tumours, motoneurone disease, Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis and peripheral neuropathies associated with diabetes, heavy metal or alcohol toxicity, renal failure and/or infectious diseases such as herpes, rubella, measles,  
25 chicken pox, HIV and/or HTLV-1. This aspect is also useful for treating neurons or glia damaged by trauma or disease.

Alternatively, the method is aimed at targeting certain cells such as cancer cells wherein expression is required of a death signal from p75<sup>NTR</sup> or a derivative,  
30 functional equivalent or homologue thereof adjacent, proximal or otherwise juxtaposed to a membrane-associating portion of p75<sup>NTR</sup> or other membrane



molecules or is in multimeric form. The nucleic acid molecule may require modification to ensure appropriate targeting to the cell or the nucleic acid molecule may be injected directly into cancerous tissue.

5 Another aspect of the present invention provides a biological composition comprising a genetic molecule capable of being expressed into a p75<sup>NTR</sup> death signal antagonist or a p75<sup>NTR</sup> death signal. The biological composition further comprises one or more pharmaceutically acceptable carriers and/or diluents. The nucleic acid molecules according to this aspect of the present invention may be  
10 naked nucleic acid molecules or contained or associated with a viral vector or other suitable delivery mechanism.

Another aspect of the present invention is directed to a biological composition comprising a molecule capable of antagonising p75<sup>NTR</sup>-mediated death signalling of  
15 a cell.

Suitable molecules according to this aspect of the present invention are as contemplated above and include a peptide, polypeptide or protein comprising a soluble form of the p75<sup>NTR</sup> death signalling region or an antagonist of a component  
20 of the p75<sup>NTR</sup> death signalling pathway.

The present invention is also useful as a culture agent such as preventing or reducing the death of cells *in vitro*. The present invention is particularly useful *in vitro* when used in combination with LIF. Even more particularly, the present  
25 invention is useful for culturing recombinant cell lines.

The present invention also provides for the use of the death signal of p75<sup>NTR</sup> in the manufacture of a medicament for the treatment of neurodegenerative diseases in animals. Preferred animals include humans, primates, livestock animals, laboratory  
30 test animals, companion animals and captive wild animals.

2

•

[illegible]

## EXAMPLE 1

The aim of this example was to determine the protein domains on p75<sup>NTR</sup> responsible for death signalling.

In order to investigate how p75<sup>NTR</sup> signals neural death, the inventors devised a robust *in vitro* assay for p75<sup>NTR</sup> induced death. Plasmid expression constructs were microinjected into individual neurons in the presence of the growth factor LIF, and the survival of the neurons expressing the different plasmids was determined. A series of plasmid constructs which encode incomplete p75<sup>NTR</sup> proteins were made (see Figure 1) and the ability of each protein to signal death when over expressed was assessed.

The p75<sup>NTR</sup> protein is a transmembrane protein comprised of a large extracellular domain with four cysteine rich motifs responsible for interacting with soluble growth factors, and a short cytoplasmic, intracellular tail. The cytoplasmic domain does not contain a kinase domain but contains a domain with significant homology to a motif known as a "death domain" [400>9, 400>10], found in apoptosis-inducing Tumour Necrosis Factor Receptors (TNFR) and TNFR-associating death-effector proteins [9].

Using expression plasmids of p75<sup>NTR</sup> proteins deleted for either the entire cytoplasmic domain (p75nc) or a significant portion of the cytoplasmic domain including the entire death domain (p75tm), the inventors found that the cytoplasmic domain is responsible for death signalling. Surprisingly, the intracellular 35 amino acid domain juxtaposed to the membrane, and not the death domain, is responsible for death signalling (Figure 2). This region of the p75<sup>NTR</sup> protein shows no homology to other death inducing proteins or to known functional motifs.

To further investigate the domain required for death signalling the inventors made

constructs expressing p75<sup>NTR</sup> proteins deleted for the extracellular domain or the extracellular and transmembrane domains. Proteins without extracellular domains retain the signal peptide which is responsible for correctly transporting the protein into the cell membrane. Proteins without transmembrane domains are expressed free in the cytoplasm of the cell and are epitope tagged with a FLAG motif for detection.

The inventors found that the extracellular domain of p75<sup>NTR</sup> had a significant inhibitory effect of the ability of the cytoplasmic domain to signal cell death.

10 Furthermore, the membrane linked 35 amino acid cytoplasmic domain (c35) was a potent stimulant of neural death with over 90% of cells injected with the plasmid dead after 16 hours (Figure 3). However, if the cytoplasmic 35 amino acid domain is not associated with the membrane, the ability of the domain to induce death is removed (Figure 4).

15

These results indicate that the domain responsible for death induction is within the first 35 amino acids of the cytoplasmic tail but that the transmembrane domain, or at least association with the membrane, is required for death-signal activation. This may be related to the ability of the transmembrane protein to more efficiently form death-signal inducing multimers, or that the position of the p75<sup>NTR</sup> protein in relation to other membrane-bound accessory molecules might be important in initiating death signalling.

The inventors hypothesised that the free cytoplasmic expressed 35 amino acid domain might be able to interfere with death signalling from full length p75<sup>NTR</sup> proteins by a dominant-negative mechanism, and attempted to inhibit the death by co-expressing the proteins. Given the results presented below regarding the ability of overexpression of Bcl-2 to enhance p75<sup>NTR</sup> killing, this paradigm was used to test the ability of the c35 protein to inhibit death signalling. The inventors found that indeed the expression of the c35 protein was able to inhibit this killing (Figure 5). This further indicates that p75<sup>NTR</sup> signals killing *via* interaction of an accessory

molecule to a motif within the first 35 amino acids of the cytoplasmic domain.

## EXAMPLE 2

The aim of this example is to determine the minimum number of amino acid  
5 residues on c35 require to mediate death signalling.

A series of deletion and truncation mutants in the c35 region are produced and  
tested for the ability to induce death signalling.

~~10 The deletion mutants from the membrane distal end are as follows:~~

~~KRWNSCKQNKQGANSRPNQTPPPEGEKLHSDSG;~~

~~KRWNSCKQNKQGANSRPNQTPPPEGEKLHSDS;~~

~~KRWNSCKQNKQGANSRPNQTPPPEGEKLHSD;~~

15 ~~KRWNSCKQNKQGANSRPNQTPPPEGEKLHS;~~

~~KRWNSCKQNKQGANSRPNQTPPPEGEKLH;~~

~~KRWNSCKQNKQGANSRPNQTPPPEGEKL;~~

~~KRWNSCKQNKQGANSRPNQTPPPEGEK;~~

~~KRWNSCKQNKQGANSRPNQTPPPEGE;~~

20 ~~KRWNSCKQNKQGANSRPNQTPPPEG;~~

~~KRWNSCKQNKQGANSRPNQTPPPE;~~

~~KRWNSCKQNKQGANSRPNQTPPP;~~

~~KRWNSCKQNKQGANSRPNQTPP;~~

~~KRWNSCKQNKQGANSRPNQTP;~~

25 ~~KRWNSCKQNKQGANSRPNQT;~~

~~KRWNSCKQNKQGANSRPNQ;~~

~~KRWNSCKQNKQGANSRPN;~~

~~KRWNSCKQNKQGANSRPV;~~

~~KRWNSCKQNKQGANSRP;~~

30 ~~KRWNSCKQNKQGANSR;~~

~~KRWNSCKQNKQGANS;~~

~~KRWNSCKQNKQGAN;~~

~~KRWNSCKQNKQGA;~~

~~KRWNSCKQNKQG;~~

~~KRWNSCKQNKQ;~~

5 ~~KRWNSCKQNK;~~

~~KRWNSCKQN;~~

~~KRWNSCKQ;~~

~~KRWNSCK;~~

~~KRWNSC;~~

10 ~~KRWNS;~~

~~KRWN;~~

~~KRW;~~

~~KR; and~~

~~K.~~

~~-15-~~

~~The deletion mutants from the membrane proximal end are as follows:~~

~~RWNSCKQNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~WNSCKQNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

20 ~~NSCKQNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~SCKQNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~CKQNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~KQNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~QNKQGANSRPVNQTPPPEGEKLHSDSGI;~~

25 ~~NKQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~KQGANSRPVNQTPPPEGEKLHSDSGI;~~

~~QGANSRPVNQTPPPEGEKLHSDSGI;~~

~~GANSRPVNQTPPPEGEKLHSDSGI;~~

~~ANSRPVNQTPPPEGEKLHSDSGI;~~

30 ~~NSRPVNQTPPPEGEKLHSDSGI;~~

~~SRPVNQTPPPEGEKLHSDSGI;~~

~~RPVNOTPPPEGEKLHSDSGI;~~

~~PVNQTPPEGEKLHSDSGI;~~

~~VNQTPPEGEKLHSDSGI;~~

~~NQTPPEGEKLHSDSGI;~~

5 QTPPPEGEKLSHSDSGI;

TPPPEGEKLHSDSGI;

~~PPPEGEKLHSDSGI;~~

~~PPEGEKLHSDSGI;~~

PEGEKLHSDSGI;

10 ~~EGEKLHSDSGI;~~

GEKLHSDSGI;

~~EKLHSDSGI;~~

~~KLHSDSGI;~~

~~LHSDSGI;~~

~~15~~ HSDSGI;

SDSGI;

DSGI;

~~SGI;~~

~~GI; and~~

20 l.

### EXAMPLE 3

#### ROLE OF BCL-2 IN PROMOTING p75<sup>NTR</sup> MEDIATED DEATH SIGNALLING

As the inventors had shown that the death of dorsal root ganglia (DRG) sensory  
5 neurons *in vitro* and *in vivo*, was, at least in part, mediated by p75<sup>NTR</sup>, p75<sup>NTR</sup> was  
over-expressed in these cells by microinjecting rat p75<sup>NTR</sup> cDNA expressing plasmid  
into the nucleus of mouse sensory neurons. These were cultured in the presence  
of the LIF to prevent neural death not linked to p75<sup>NTR</sup> mechanisms. It was found  
that the expression of the rat p75<sup>NTR</sup> could be detected by surface  
10 immunofluorescence within 24 hours of injection. The injected neurons were  
observed over a 48 hour period and the viability was assessed. It was found that  
within the first 16 hours, a significantly higher number of p75<sup>NTR</sup> plasmid injected  
neurons had died compared to neurons injected with control plasmids  $\beta$ -  
galactosidase, or a truncated p75<sup>NTR</sup> protein lacking the entire cytoplasmic domain  
15 (p75<sup>NTR</sup>nc). It was found that p75<sup>NTR</sup>-mediated neural death occurred later in the  
experiment similar to Fas/TNF-induced rapid cell death. Since both full-length  
p75<sup>NTR</sup> and p75<sup>NTR</sup>nc protein showed a similar level of expression after injection, this  
indicates that the cytoplasmic domain of p75<sup>NTR</sup> is required for death signalling.  
This was expected since the cytoplasmic tail contains a sequence with homology to  
20 the Fas/TNFR "death domain" [9].

The inventors next examined whether deletion of the "death domain" also abolished  
the ability of p75<sup>NTR</sup> to kill. It was found that the neural death observed after  
expression of p75<sup>NTR</sup> with a truncated cytoplasmic tail (p75<sup>NTR</sup>tr) was equivalent to  
25 the full-length p75<sup>NTR</sup> protein. This demonstrated that the "death domain" was not  
required for p75<sup>NTR</sup> killing and, since the p75<sup>NTR</sup> death domain has recently been  
shown to have a different tertiary structure to TNFR family death domain and does  
not self-associate *in vitro*, it suggests that the p75<sup>NTR</sup> "death domain" may not  
normally function to induce death. Together, these results predict that an  
30 alternative pathway involving proteins other than "death domain" adapter proteins,  
such as TRADD and FADD, is responsible for p75<sup>NTR</sup>-mediated killing.



The Bcl-2 family of proteins is involved in mediating apoptotic signalling pathways, and can homodimerise or heterodimerise with other family members. Bcl-2 and Bcl-xL are well characterised inhibitors of stress-induced apoptosis, JNK activation and neural death due to growth-factor limitation. However, both are poor inhibitors of Fas and TNFR mediated apoptosis. As it had been shown previously that high levels of Bcl-2 or Bcl-xL blocked neural cell death in a variety of models, the inventors examined whether over-expression of these proteins could block the death induced by p75<sup>NTR</sup>.

10 The inventors found that over-expression of Bcl-xL protected neurons against p75<sup>NTR</sup>-induced death, supporting the hypothesis that p75<sup>NTR</sup> signals through an alternative pathway to TNFR-induced apoptosis. In contrast, while Bcl-2 over-expression alone had no effect on cell survival in the presence of LIF, Bcl-2 in combination with p75<sup>NTR</sup> over-expression, surprisingly, induced a significant increase in neural death above that seen with p75<sup>NTR</sup> over-expression alone. Bcl-2 in combination with p75<sup>NTR</sup>nc did not cause significant cell death and furthermore, the cell death observed with p75<sup>NTR</sup> and Bcl-2 over-expression was totally ablated if the cells were cultured in NGF. Bcl-2 was able to protect against neural death induced by NGF withdrawal, but not withdrawal of LIF. Thus, at the same expression levels in the same neural population, Bcl-2 was able to prevent or enhance neural cell death depending on the nature of the death signal.

These results are surprising since Bcl-2 has previously been shown to have similar actions to Bcl-xL in almost all cell-death systems.

25

To determine whether the paradoxical effect of Bcl-2 on p75<sup>NTR</sup>-induced killing was related to its known anti-apoptotic activity, Bcl-2 proteins with inactivating point mutation, G145E, in the "Bcl-2 Homology" BH1 domain and W188A in the BH2 domain were utilised. Like wildtype Bcl-2, expression of either Bcl-2 mutant alone did not effect neural survival. In combination with p75<sup>NTR</sup> expression, the enhanced killing effect seen with Bcl-2 co-expression was abrogated by the G145E mutation,

even though the proteins were expressed to comparable levels. Thus, an intact BH1 homology region is required for the death promoting activity of Bcl-2.

Mutation of the equivalent G138 residue in Bcl-xL results in a conformational  
5 change between  $\alpha$ -helices 4 and 5, disrupting access to the hydrophobic cleft  
formed by BH1, BH2 and BH3 domains. Therefore, the molecular mechanism by  
which Bcl-2 participates in the p75<sup>NTR</sup> killing pathway may be dependent on  
interactions either directly with the BH domains or with the hydrophobic cleft, as  
indicated with experiments using the W188A mutation. Co-expression of p75<sup>NTR</sup>  
10 with the Bcl-2 W188A protein not only abrogated the increased p75<sup>NTR</sup> killing but,  
more importantly, protected neurons from any p75<sup>NTR</sup>-induced death, reminiscent of  
that seen with Bcl-xL. These experiments suggest that the conformation of the Bcl-  
2 protein is integral to the opposing functions observed herein.

15 The inventors had observed that DRG neurons isolated from newborn mice  
depleted for p75<sup>NTR</sup> were less susceptible to NGF withdrawal, as is the case with  
sympathetic neurons, when compared to neurons from wildtype mice. This is  
indicative of absent or delayed naturally occurring cell death observed in these  
mice. The inventors attempted to induce cell death in p75<sup>NTR</sup> "knock out" DRG  
20 neurons by re-introducing p75<sup>NTR</sup> expression. Surprisingly, apoptosis was not  
induced by re-expression into "knock out" DRG neurons, the inventors found that  
neural death was significantly increased under these conditions. This implicated an  
absolute requirement for Bcl-2 in mediating p75<sup>NTR</sup> killing.

25 The inventors tested, therefore, whether high endogenous Bcl-2 levels might be  
necessary for successful p75<sup>NTR</sup>-mediated killing in normal neurons by assaying  
p75<sup>NTR</sup> killing in Bcl-2 depleted cells. Endogenous Bcl-2 was down regulated by  
antisense as previously described. When the Bcl-2 antisense plasmid was injected  
at the same time as p75<sup>NTR</sup> plasmids no diminishment in the death signal was seen.  
30 If, however, the Bcl-2 antisense was microinjected first (to give time to reduced Bcl-  
2 production and deplete endogenous Bcl-2; and then a day later the p75<sup>NTR</sup> or

p75<sup>NTR</sup>nc constructs were microinjected, there was no difference in survival between p75<sup>NTR</sup> and p75<sup>NTR</sup>nc expressing neurons, strongly suggesting that endogenous Bcl-2 is required for p75<sup>NTR</sup> killing effects. To confirm this observation, the inventors isolated neurons from newborn Bcl-2 "knock out" mice (an  
5 heterozygous line of mice containing a disrupted Bcl-2 gene) and their wild-type litter mates and compared the effect of p75<sup>NTR</sup> over-expression with control plasmid p75<sup>NTR</sup>. It was found that the neurons isolated from Bcl-2 deficient mice were significantly protected from p75<sup>NTR</sup> killing, showing a 56.9% (n=3) reduction in death compared wildtype neurons, supporting the hypothesis that endogenous Bcl-2 is  
10 required for p75<sup>NTR</sup> killing.

Bcl-2 has previously been observed to increase cell death when highly expressed both *in vitro* and *in vivo* when expressed at high levels as a transgene, causing increased apoptosis in the brain under a neuron specific promoter, or in  
15 photoreceptor cells when expressed specifically under a rhodopsin promoter. Thus, it is possible that the high level of Bcl-2 is able to "prime" the death pathway such that an apoptotic stimulus *via* p75<sup>NTR</sup> results in rapid cell death.

Bcl-2 and Bcl-xL when cleaved by caspases have also been shown to be capable  
20 of promoting apoptosis *in vitro*, with cells expressing non-cleavable mutant Bcl-2 and Bcl-xL proteins showing increased viability compared to cells expressing wildtype proteins. Cleavage of Bcl-2 is possible in this system, however, the Bcl-2 mutations which results in loss of death promoting activity, would not prevent cleavage of Bcl-2, indicating that cleavage of Bcl-2 would only be part of the  
25 mechanism by which Bcl-2 promotes killing. In addition, if cleavage was the dominant mechanism, Bcl-xL might be expected to act as a death signalling protein in this system.

To investigate whether the p75<sup>NTR</sup>-Bcl-2 death-signalling cascade was dependent  
30 on caspase activation, inhibitors of caspases were employed. In the presence of z-VAD, a nonspecific caspase peptide inhibitor, or after co-expression of modified

crmA plasmids, designed to inhibit Group II caspases such as caspases 2 and 3, p75<sup>NTR</sup>-mediated death was significantly reduced. Similarly, the modified crmA was able to block the killing induced by co-expression of p75<sup>NTR</sup> and Bcl-2. This indicates that p75<sup>NTR</sup> induced apoptosis is a caspase dependent pathway and that  
5 the mechanism by which Bcl-2 assists killing is through the same pathway.

#### EXAMPLE 4

#### ANTAGONISM OF p75<sup>NTR</sup> MEDIATED DEATH SIGNALLING

10 ~~Figure 6 shows that soluble c35 (35mer) [ $\langle 400 \rangle 7$  and  $\langle 400 \rangle 8$ ] protects cells from~~  
11 ~~death signalling in a dose-dependent manner against membrane bound c35.~~  
12 ~~Furthermore, the 35mer when expressed from a genetic construct, protected~~  
13 ~~Schwann cells against NGF-induced death. c35 when expressed in soluble form~~  
14 ~~can also protect cells against membrane bound c35. The inventors show in Figure~~  
15 ~~7 that soluble c35 can also protect against membrane-linked, expressed c35. A~~  
16 ~~truncated form of c35, a 29mer [ $\langle 400 \rangle 11$  and  $\langle 400 \rangle 12$ ], also protected against~~  
17 ~~membrane-bound c35, when in soluble form.~~

Figure 8 shows that the 29mer with a palmitoyl group at the membrane (amino) end  
20 resulted in cell death. The palmitoylation links the peptide to the plasma  
membrane. This membrane-linked 29mer leads to cell death whereas its soluble  
form protects cells against p75<sup>NTR</sup>-mediated death signalling.

#### EXAMPLE 5

#### EFFECTS OF PALMITOYLATION

25 Figure 9 shows the effects of palmitoylated 29 mer (fused to penetratin) on  
mediating cell death. Cells were washed with peptide (2  $\mu$ M) for approximately 110  
minutes and the cells were then washed. Controls included penetratin-fused 29  
30 mer, palmitoylated penetratin-fused 29 mer palmitoylated penetratin-fused gp130  
and penetratin alone. Palmitoylated, penetratin fused 29 mer mediated significant

cell death.

**EXAMPLE 6**  
**PASSAGE ACROSS BLOOD BRAIN BARRIER**

5

The ability for peptides to cross the blood brain barrier is tested using fluorescence-linked peptides injected intraperitoneally into mice. The peptides may be fused to penetratin or fused or associated with the TAT protein from HIV (18).

10

**EXAMPLE 17**  
**ANIMAL MODELS**

Peptides are delivered with penetratin or TAT (18) to various animal models for neurodegenerative diseases. The animal models used include:

15

Animal Model	Disease
Axotomy of newborn rat sensory neurons	Peripheral neuropathies
Axotomy of newborn rat motor neurons (SOD1 mice: B6SJL-TgN [SOD1-G93A] 1 Gurd1)	Motor neuron disease
Ischemia of adult rats	Stroke
Experimental allergic encephamylitis and optic nerve axotomy [19]	Multiple sclerosis

25

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and  
30 modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or

collectively, and any and all combinations of any two or more of said steps or features.

[illegible]

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